PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 :
C07K 15/00, G01N 33/576, C12N 15/40, 15/51, 5/10, C12P 21/08, C12Q 1/70, A61K 39/29

A1

(11) International Publication Number: WO 94/13700

(43) International Publication Date: 23 June 1994 (23.06.94)

(21) International Application Number: PCT/EP93/03478

(22) International Filing Date: 7 December 1993 (07.12.93)

(30) Priority Data:
92203802.1 7 December 1992 (07.12.92) EP
(34) Countries for which the regional or international application was filed: NL et al.
93201854.2 25 June 1993 (25.06.93) EP
(34) Countries for which the regional or international application was filed: NL et al.

(71) Applicant *(for all designated States except US)*: AKZO NOBEL N.V. [NL/NL]; Velperweg 76, NL-6824 BM Arnhem (NL).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: HABETS, Winand, Johannes, Antonius [NL/NL]; 2e Hervendreef 55, NL-5232 JB Den Bosch (NL). BOENDER, Pieter, Jacob [NL/NL]; Ubbergseweg 58, NL-6522 KJ Nijmegen (NL).

(74) Agent: HERMANS, Franciscus, G. M.; Postbus 20, 5340 BH OSS (NL).

(81) Designated States: AU, CA, FI, JP, KR, US, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: PEPTIDES FROM THE C33 REGION OF HCV, ANTIBODIES THERETO AND METHODS FOR THE DETECTION OF HCV

(57) Abstract

The invention concerns peptides which react immunochemically with antibodies directed against HCV. A preferred peptide according to the invention comprises an HCV specific epitope of the NS-3 protein. The invention further relates to antibodies that specifically react with the NS-3 protein of HCV. Methods for the detection of HCV or HCV antibodies, and a method for the detection of antibodies specifically reactive with the NS-3 antigen are also part of the present invention.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	**GB**	United Kingdom	**MR**	Mauritania
AU	Australia	**GE**	Georgia	**MW**	Malawi
BB	Barbados	**GN**	Guinea	**NE**	Niger
BE	Belgium	**GR**	Greece	**NL**	Netherlands
BF	Burkina Faso	**HU**	Hungary	**NO**	Norway
BG	Bulgaria	**IE**	Ireland	**NZ**	New Zealand
BJ	Benin	**IT**	Italy	**PL**	Poland
BR	Brazil	**JP**	Japan	**PT**	Portugal
BY	Belarus	**KE**	Kenya	**RO**	Romania
CA	Canada	**KG**	Kyrgystan	**RU**	Russian Federation
CF	Central African Republic	**KP**	Democratic People's Republic of Korea	**SD**	Sudan
CG	Congo			**SE**	Sweden
CH	Switzerland	**KR**	Republic of Korea	**SI**	Slovenia
CI	Côte d'Ivoire	**KZ**	Kazakhstan	**SK**	Slovakia
CM	Cameroon	**LI**	Liechtenstein	**SN**	Senegal
CN	China	**LK**	Sri Lanka	**TD**	Chad
CS	Czechoslovakia	**LU**	Luxembourg	**TG**	Togo
CZ	Czech Republic	**LV**	Latvia	**TJ**	Tajikistan
DE	Germany	**MC**	Monaco	**TT**	Trinidad and Tobago
DK	Denmark	**MD**	Republic of Moldova	**UA**	Ukraine
ES	Spain	**MG**	Madagascar	**US**	United States of America
FI	Finland	**ML**	Mali	**UZ**	Uzbekistan
FR	France	**MN**	Mongolia	**VN**	Viet Nam
GA	Gabon				

Title: Peptides from the C33 region of HCV, antibodies thereto and methods for the detection of HCV.

The invention relates to peptides which react immunochemically with antibodies directed against the Hepatitis C virus and to nucleic acid sequences encoding these peptides.

The invention also relates to methods for the detection of HCV or anti-HCV in a test fluid and to immunochemical reagents and a test kits for carrying out said detection methods.

Hepatitis C virus (HCV) is a 9.4-kb, single stranded polyadenylated RNA virus which has been recognized as one of the causative agents of NANB hepatitis (Non-A, Non-B). It causes acute and chronic liver disease and is implicated in hepatocellular carcinoma.

It can be distinguished from other forms of viral-associated liver diseases, including those caused by known hepatitis viruses, i.e., hepatitis A virus (HAV), hepatitis B virus (HBV), and hepatitis delta virus (HDV), as well as the hepatitis induced by cytomegalovirus (CMV) or Epstein-Barr virus (EBV). Evidence based on hydrophobicity plots and sequence homologies suggests that HCV may be distantly related to the family Flaviviridae (Houghton M. et al., Hepatology, 14:381, 1991). Non-A, Non-B Hepatitis was first identified in transfused individuals. Transmission from man to chimpanzee and serial passage in chimpanzees provided evidence that Non-A, Non-B Hepatitis is due to a transmissible infectious agent or agents.

Epidemiologic evidence is suggestive that three types of Non-A, Non-B Hepatitis exist: the

water-borne epidemic type; the blood or needle associated type; and the sporadically occurring (community acquired) type. The viral genome of HCV encodes a polyprotein of approximately 3010 amino acids that undergoes extensive posttranslational processing. The viral structural region is located upstream from the nonstructural region and putatively includes a highly conserved 19-kDa nucleocapsid protein, and two extensively glycosylated envelope polypeptides, gp 33 (E1) and gp72 (E2/NS1). Recent studies indicate that substantial sequence heterogeneity exists among virtually all HCV isolates in the N-terminal region of E2/NS1, suggesting that this region of the HCV envelope may be under strong immune selection. A variety of presumed nonstructural proteins are processed from the remainder of the HCV polyprotein, including a membrane-bound 23-kDa protein, NS2, and a soluble protein of approximately 60 kDa, NS3, which corresponds to the viral helicase and may contain a N-terminal serine protease domain, currently thought to be involved in the processing of the NS proteins. The function of the NS4 protein is presently unknown, but it comprises the 5-1-1 fragment that contains immunodominant antibody binding sites (Kuo G. et al., Science 244:362, 1991; Cerino A. et al.J.Immunol., 147:2692) ; NS5 contains the viral replicase. Clinical studies have shown that, following exposure to HCV, antibodies to conserved regions of the viral nucleoprotein and NS3 may appear several weeks before seroconversion to anti-c100-3, a recombinant protein encompassing the C-terminus of NS3 and part of the NS4 protein.

Thus, serological assays incorporating the highly-conserved HCV nucleocapsid protein as well as NS3 are likely to become useful diagnostic markers of acute HCV infection.

For the development of a specific and sensitive method to enable a reliable diagnosis to be made in various phases of the infection with HCV it is of great importance to identify immunodominant viral epitopes of this type.

An objective of the current invention is to provide small peptides useful for the diagnosis and monitoring of HCV infection.

Long recombinant antigens encoding at least part of the putative HCV NS3 antigen are reactive with antibodies against HCV but have substantial disadvantages as outlined above. Small synthetic peptides circumvent these disadvantages but are not satisfactory immunoreactive. The objective of present invention is to provide peptides with a length small enough to have the advantages of small synthetic peptides but at the same time large enough to be immunoreactive with antibodies against HCV.

Smaller peptides (12-mers) from the region encoded by the putative HCV NS3-gene were found to be not particularly useful to detect antibodies against HCV. Larger polypeptides are unpractical because they cannot be easily expressed as fusion proteins, they are prone to endogenous proteolysis and there are increased chances on false positive reactivities. Larger polypeptides are also difficult to synthesize, difficult to purify and might be infectious.

In this invention, regions of the HCV NS3-genome are identified which form an optimal synthesis between length and immunoreactivity.

For the screening of blood-sera on the presence of antibodies to antigens of the Hepatitis C virus four regions of the HCV genome currently seem important for use in screening assays. These antigens are the core antigen, the NS-3 antigen, NS-5 antigen and the NS-4 antigen. Recent studies, (Lelie N., Cuypers T., Zaayer H., Bresters D., to be published) indicate that, of 1100 sera from blood donors, that were HCV positive in a screening assay, about 50% are indeterminate in RIBA (Recombinant immunoblot assay), which means that these sera do not recognize all (in fact recognize only one) of the above mentioned antigens. These sera, although containing antibodies that react with one of the above mentioned antigens, are not always infected with the Hepatitis C virus. On the basis of the tests that are presently used by blood banks for the screening of donor blood, these sera would be considered infected, and would therefore be considered useless for transfusion purposes, while most of them may not be infected with HCV at all. The need therefore exists for a reliable and accurate confirmation test that will discriminate between all HCV infected and non-infected sera.

About 5 % (51 sera) from the 1100 screening-positive sera recognize only a part of the NS-3 region, the C33 antigen. These sera will be referred to in this application as "C33-only" sera, since the C-33 antigen is the only antigen recognized.

To determine whether these sera are actually infected, an assay can be performed based on a nucleic acid amplification technique like the Polymerase Chain Reaction (PCR), to detect the

presence of HCV derived nucleic acid. Using this technique it has been found that only a small percentage of the C-33 only sera is actually infected with HCV. The reactivity of PCR negative C-33 only sera with the C-33 antigen can be explained by the presence of a cross-reacting epitope on the C-33 antigen. Although a PCR test is a reliable method for the detection of the presence of HCV in C-33 only sera, it is a rather laborious procedure. If a screening test could be developed that would discriminate between all infectious and non-infectious sera, the need for a separate PCR test would be eliminated. For this purpose, a non-cross reacting NS-3 antigen would be necessary, that is an antigen that comprises the C-33 epitope responsible for the true anti-HCV NS-3 immune response, in which the cross-reacting epitope is no longer present.

It is a further object of the present invention to provide a peptide comprising a HCV specific NS-3 antigenic sequence.

The present invention includes peptides with amino acid sequences selected from the group of sequences depicted in SEQ ID No.: 6, 7, 8, 9 and 10 and combinations thereof or fragments of said group of sequences or analogues of said group of sequences which are immunochemically reactive with HCV-antibodies.

A library can be constructed consisting of DNA fragments from a recombinant clone encoding an antigen which covers most if not all of the putative HCV NS3 gene. These fragments could range in size from approximately 50 to 300 nucleotides and when expressed in the appropriate reading frame encoded HCV polypeptides ranging

from approximately 17 to 100 amino acids. In this way a library can be constructed containing enough different recombinants to ensure that any possible fragment in the range of 17 to 100 amino acids is contained at least once. Recombinants which express exceptionally reactive antigens can be selected using an appropriate antibody as a probe and DNA sequence encoding the exceptionally reactive peptides.

The peptides according to the invention are located in the putative NS3 region of the HCV genome.

The peptides according to the invention have been found to be exceptionally immunochemically reactive with HCV-antibodies. An advantage of this reactivity is that the use of one or more of the peptides according to the invention will increase the specificity of the immunological assay when compared to the use of large recombinant fragments. Another advantage is that the use of one or more of the peptides will increase the sensivity of the immunological assay.

The invention also comprises fragments of said peptides which are still immunochemically reactive with HCV-antibodies.

The term "fragment" as used herein means an amino acid sequence comprising a subsequence of a peptide of the invention. Said fragment is a peptide having one or more immunogenic determinants of the HCV NS3-antigen.

The invention comprises, but is not limited to specific peptides comprising fragments according to the invention with the amino acid sequence as depicted in SEQ.ID. 7.

These peptides comprise an amino acid sequence as depicted in SEQ. ID. 6, 8, 9 and 10.

It is evident that other fragments of the peptides according to the invention having the immunochemical reactivity with HCV patient sera are also part of this invention.

Fragments can inter alia be produced by enzymatic cleavage of precursor molecules, using restriction endonucleases for the DNA and proteases for the polypeptides. Other methods include chemical synthesis of the fragments or the expression of polypeptide fragments by DNA fragments.

Analogues or derivatives of the peptides according to SEQ ID No. 6-10 are also included in the invention.

The term "analogues" refers for instance to post-expression modifications of a peptide, for example, glycosylations, acetylations, phosphorylations etc.

Without specifically being incorporated in the claims, it is self-evident that several amino acids in the peptides according to the invention can be deleted or inserted or substituted by other amino acids or amino acid analogues or derivatives due to strain-to-strain variations among different isolates of HCV without affecting the immunochemical activity of the peptides in question.

In addition, with analogues of these peptides are also meant acid addition salts of the peptides, amides of the peptides and specifically the C-terminal amides, esters and specifically C-terminal esters and N-acyl derivatives specifically N-terminal acyl derivatives and in particular N-acetyl derivatives.

The preparation of the peptides according to the invention can be effected adapting one of the known organic chemical methods for peptide synthesis or with the aid of recombinant DNA techniques. This latter method involves the preparation of the desired peptide by means of expressing a recombinant polynucleotide with the aid of a suitable vector containing a polynucleotide sequence which is coding for one or more of the peptides in question and introducing the vector in a suitable host.

The organic chemical methods for peptide synthesis are considered to include the coupling of the required amino acids by means of a condensation reaction, either in homogeneous phase or with the aid of a so-called solid phase. The condensation reaction can be carried out as follows:

a) condensation of a compound (amino acid, peptide) with a free carboxyl group and protected other reactive groups with a compound (amino acid, peptide) with a free amino group and protected other reactive groups, in the presence of a condensation agent;

b) condensation of a compound (amino acid, peptide) with an activated carboxyl group and free or protected other reaction groups with a compound (amino acid, peptide) with a free amino group and free or protected other reactive groups.

Activation of the carboxyl group can take place, inter alia, by converting the carboxyl group to an acid halide, azide, anhydride, imidazolide or an activated ester, such as the N-hydroxy-succinimide, N-hydroxy-benzotriazole or p-nitrophenyl ester.

The most common methods for the above condensation reactions are: the carbodiimide method, the azide method, the mixed anhydride method and the method using activated esters, such as described in The Peptides, Analysis, Synthesis, Biology Vol. 1-3 (Ed. Gross, E. and Meienhofer, J.) 1979, 1980, 1981 (Academic Press, Inc.).

Alternatively, the peptides according to the invention are prepared with the aid of recombinant DNA techniques.

Peptides can, for example, be incorporated in a repeating sequence ("in tandem") or can be prepared as a constituent of a (much larger) protein or polypeptide. For this purpose, as a constituent of a recombinant DNA, a polynucleotide with a specific nucleic acid sequence can be used which codes for the peptide according to the invention.

A polynucleotide of this type, which is coding for the peptide according to the invention, and a recombinant DNA in which this polynucleotide is incorporated likewise fall within the scope of the invention.

The present invention is further directed to a peptide comprising a HCV specific epitope of the NS-3 antigen of the Hepatitis C virus, preferably comprising the amino acid sequence as depicted in SEQ ID No: 7.

It has been shown that this peptide comprises an epitope of the NS-3 antigen that will react with antibodies that are specifically directed against HCV.

This peptide can therefore be used in an screening test for the detection of HCV, to

eliminate, or at least minimize, the cross reactivity with non-HCV antibodies. C33-only sera that would recognize this peptide are therefore infected with the HCV virus. It is a great advantage of the use of this particular peptide in assays (combined with other HCV antigens) that no additional PCR-based assay is needed to determine whether a C33-only serum is actually infected with HCV.

Of course an immuno assay based on the use of this peptide can also be used to replace the PCR tests presently used to discriminate between infected and non-infected C33 only sera.

The invention also relates to an immunochemical reagent, which reagent comprises at least one of the peptides.

An "immunochemical reagent" according to the invention may comprise one or more peptides according to the invention and a suitable support or a labelling substance.

Supports which can be used are, for example, the inner wall of a microtest well or a cuvette, a tube or capillary, a membrane, filter, test strip or the surface of a particle such as, for example, a latex particle, an erythrocyte, a dye sol, a metal sol or metal compound as sol particle, a carrier protein such as BSA or KLH.

Labelling substances which can be used are, inter alia, a radioactive isotope, a fluorescent compound, an enzyme, a dye sol, metal sol or metal compound as sol particle.

The invention further encompasses nucleic acid sequences encoding the peptides according to the invention preferably a nucleic acid sequence

containing at least part of the DNA sequence shown in SEQ ID No. 1, 2, 3, 4, and 5.

"Nucleic acid sequence" as used herein refers to a polymeric form of nucleotides of any length, both to ribonucleic acid sequences and to deoxyribonucleic acid sequences. In principle, this term refers to the primary structure of the molecule. Thus, this term includes double and single stranded DNA, as well as double and single stranded RNA, and modifications thereof.

A nucleic acid sequence according to the present invention can be ligated to various replication effecting DNA sequences with which it is not associated or linked in nature resulting in a so called recombinant vector molecule which can be used for the transformation of a suitable host. Useful recombinant vector molecules, are preferably derived from, for example plasmids, bacteriophages, cosmids or viruses.

Specific vectors or cloning vehicles which can be used to clone nucleic acid sequences according to the invention are known in the art and include inter alia plasmid vectors such as pBR322, the various pUC, pGEM and Bluescript plasmids, bacteriophages, e.g. kgt-Wes, Charon 28 and the M13 derived phages or viral vectors such as SV40, adenovirus or polyoma virus (see also Rodriquez, R.L. and D.T. Denhardt, ed., Vectors: A survey of molecular cloning vectors and their uses, Butterworths, 1988; Lenstra, J.A. et al., Arch. Virol. 110, 1-24, 1990). The methods to be used for the construction of a recombinant vector molecule according to the invention are known to those of ordinarily skill in the art and are inter alia set forth in Maniatis, T. et al.

(Molecular Cloning A Laboratory Manual, second edition; Cold Spring Harbor Laboratory, 1989).

For example, the insertion of the nucleic acid sequence according to the invention into a cloning vector can easily be achieved when both the genes and the desired cloning vehicle have been cut with the same restriction enzyme(s) as complementary DNA termini are thereby produced.

The recombinant vector molecules according to the invention may additionally contain one or more marker activities that may be used to select for desired transformants, such as ampicillin and tetracycline resistance in pBR322, as for example ampicillin resistance and α-peptide of ß-galactosidase in pUC8.

The invention also comprises (a) host cell(s) transformed with a nucleic acid sequence or recombinant expression vector molecule described above, capable of producing the peptides according to the invention by expression of the corresponding nucleic acid sequence.

A suitable host cell is a microorganism or cell which can be transformed by a nucleic acid sequence encoding a polypeptide or by a recombinant vector molecule comprising such a nucleic acid sequence and which can if desired be used to express said polypeptide encoded by said nucleic acid sequence. The host cell can be of procaryotic origin, e.g. bacteria such as Escherichia coli, Bacillus subtilis and Pseudomonas species; or of eucaryotic origin such as yeasts, e.g. Saccharomyces cerevisiae or higher eucaryotic cells such as insect, plant or mammalian cells, including HeLa cells and Chinese hamster ovary (CHO) cells. Insect cells include

the Sf9 cell line of Spodoptera frugiperda (Luckow et al., Bio-technology 6, 47-55, 1988). Information with respect to the cloning and expression of the nucleic acid sequence of the present invention in eucaryotic cloning systems can be found in Esser, K. et al. (Plasmids of Eukaryotes, Springer-Verlag, 1986).

In general, prokaryotes are preferred for the construction of the recombinant vector molecules useful in the invention. For example E.coli K12 strains are particularly useful such as DH5α or MC1061k.

For expression nucleic acid sequences of the present invention are introduced into an expression vector, i.e. said sequences are operably linked to expression control sequences. Such control sequences may comprise promoters, enhancers, operators, inducers, ribosome binding sites etc. Therefore, the present invention provides a recombinant vector molecule comprising a nucleic acid sequence encoding the peptides identified above operably linked to expression control sequences, capable of expressing the DNA sequences contained therein in (a) transformed host cell(s).

It should, of course, be understood that the nucleotide sequences inserted at the selected site of the cloning vector may include only a fragment of the complete nucleic acid sequence encoding for the peptides according to the invention as long as the transformed host will produce a polypeptide having at least one or more immunogenic determinants .

When the host cells are bacteria, illustrative useful expression control sequences include the Trp promoter and operator (Goeddel, et al., Nucl. Acids Res. 8, 4057, 1980); the lac

promoter and operator (Chang, et al., Nature 275, 615, 1978); the outer membrane protein promoter (Nakamura, K. and Inouge, M., EMBO J. 1, 771-775, 1982); the bacteriophage k promoters and operators (Remaut, E. et al., Nucl. Acids Res. 11, 4677-4688, 1983); the α-amylase (B. subtilis) promoter and operator, termination sequence and other expression enhancement and control sequences compatible with the selected host cell. When the host cell is yeast, illustrative useful expression control sequences include, e.g., α-mating factor. For insect cells the polyhedrin or p10 promoters of baculoviruses can be used (Smith, G.E. et al., Mol. Cell. Biol. 3, 2156-65, 1983). When the host cell is of mammalian origin illustrative useful expression control sequences include, e.g., the SV-40 promoter (Berman, P.W. et al., Science 222, 524-527, 1983) or, e.g. the metallothionein promoter (Brinster, R.L., Nature 296, 39-42, 1982) or a heat shock promoter (Voellmy et al., Proc. Natl. Acad. Sci. USA 82, 4949-53, 1985). Alternatively, also expression control sequences present in HCV may be applied. For maximizing expression, see also Roberts and Lauer (Methods in Enzymology 68, 473, 1979).

Novel monoclonal antibodies that specifically react with the NS3 protein of the HCV virus and are particularly useful in immunodiagnostic tests for the detection of the presence or absence of HCV in clinical specimen are described in the co-pending and co-owned application No. EP 92.204.107.4, the contents of which are incorporated herein by reference.

In this co-pending patent application monoclonal antibodies are described which bind to

an epitope of the NS3 protein of hepatitis C virus, which epitope is recognized by monoclonal antibodies secreted by the Epstein-Barr virus-transformed human lymphocyte-B cell line deposited at the European Collection of Animal Cell Cultures, Porton Down (UK) under deposit No. 92121609.

A preferred monoclonal antibody described in this co-pending application is secreted by the Epstein-Barr virus-transformed human lymphocyte-B cell line deposited at the European Collection of Animal Cell Cultures, Porton Down (UK) under deposit No. 92121609. HCVHU.OT3 has been deposited at the ECACC on 16 December 1992, under the terms and conditions of the Budapest treaty, 1977.

This monoclonal antibody (HCVHU.OT3) recognizes peptides according to the invention comprising the sequence as depicted in SEQ ID No. 7. Using this antibody it was confirmed by the inventors that peptides according to the present invention contain a HCV specific epitope of the NS-3 antigen.

The present invention is further directed to a method for the detection of antibodies directed against HCV in a test fluid, wherein a peptide according to the invention is brought into contact with the test fluid and the presence of immune complexes formed between the peptide and antibodies in the test fluid is detected.

The presence of immune complexes formed between the peptide and antibodies in the test fluid is detected and by this detection the presence of antibodies to HCV in the test fluid is known and can be determined.

Depending on the nature and further characteristics of the immunochemical reagents used the immunochemical reaction that takes place can be a so called sandwich reaction, an agglutination reaction, a competition reaction or an inhibition reaction.

A particularly suitable method for the detection of HCV in a test fluid is based on a competition reaction between a peptide according to the invention provided with a labelling substance and a HCV antigen (present in the test fluid) whereby the peptide and the antigen are competing with the antibody directed against HCV attached to a solid support. The antibody coated on the support can, for example be an antibody according to the invention.

The invention is further directed to a method for the detection of Hepatitis C virus in a sample comprising contacting the sample with a monoclonal antibody according to the invention, and detecting immune complexes formed between the monoclonal antibody and a Hepatitis C antigen.

Carrying out, for instance, a sandwich reaction for the detection of HCV in a test sample the test kit to be used comprises a monoclonal antibody according to the invention coated on a solid support, for example the inner wall of a microtest well, and either a labelled monoclonal antibody or fragment thereof as conjugate.

A further example of an immuno assay that can be used for the detection of HCV is a inhibition assay using human monoclonal antibodies as labelled reagent. The binding of this reagent to antigen on a solid phase can be competed by antibodies in the test sample.

As already mentioned monoclonal antibodies according to the invention are very suitable in diagnosis, while those antibodies which are neutralizing are very useful in passive immunotherapy.

Part of the present invention is also a method for the detection of antibodies directed against a HCV specific epitope of the NS-3 protein of the Hepatitis C virus in a test-fluid, wherein a peptide according to the invention, comprising the amino acid sequence selected from the group of sequences depicted in SEQ.ID. 6-10, and combinations thereof or fragments of said group of sequences or analogues of said group of sequences which are immunochemically reactive with HCV antibodies, is brought into contact with the test fluid and the presence of immune complexes formed between the peptide and the antibodies in the test fluid is detected.

With this method according to the invention the need for a PCR test to detect whether C33-only sera are actually infected is no longer necessary. With the present invention a simple and accurate method for the detection of antibodies directed against a HCV specific epitope of the NS-3 protein is provided.

Antibodies directed against a HCV specific epitope of the NS-3 protein can be detected in different ways. Using an antibody as described in our co-pending application and a peptide comprising the amino acid sequence selected from the group of sequences depicted in SEQ.ID. 6-10, and combinations thereof or fragments of said group of sequences or analogues of said group of sequences which are immunochemically reactive with HCV antibodies, an inhibition or competition

test may also be designed. Part of the present invention is therefore also a method for the detection of antibodies directed against a HCV specific epitope of the NS-3 protein of the Hepatitis C virus in a test-fluid, wherein a peptide according to the invention, comprising the amino acid sequence selected from the group of sequences depicted in SEQ.ID. 6-10, and combinations thereof or fragments of said group of sequences or analogues of said group of sequences which are immunochemically reactive with HCV antibodies, coated on a suitable support, is brought into contact with the test fluid and with an antibody according to the invention, provided with a label and any label bound to the solid phase is detected.

The invention also relates to a test kit for carrying out an immuno-assay, said test kit containing at least an immunochemical reagent according to the invention.

A test kit according to the invention comprises as an essential constituent an immunochemical reagent as described above. This immunochemical reagent may comprise an antibody according to our co-pending application or a peptide according to the invention. Test kits comprising a combination of different immunochemical reagents according to the invention, for example a peptide coated on a solid support and an antibody provided with a label, are of course within the scope of this invention.

Carrying out a sandwich reaction, for the detection of HCV antibodies the test kit may comprise, for example, a peptide according to the invention coated to a solid support, for example

the inner wall of a microtest well, and either a labelled peptide according to the invention or a labelled anti-antibody. Another sandwich reaction test format is the detection of HCV antigen whereby monoclonal antibodies according to the invention are coated to a solid support and monoclonal antibodies are used as conjugate.

For instance sandwich reactions are described in our American patents with regard to the enzyme immuno assay viz. RE 31.006 and RE 32.696 (Schuurs et al.).

For carrying out a competition reaction, the test kit may comprise a peptide according to the invention coated to a solid support, and a labelled antibody directed against HCV preferably a monoclonal antibody directed against said peptide.

In an agglutination reaction the test kit comprises an immunochemical reagent which may comprise a peptide according to the invention coated to particles or sols.

Another embodiment of a test kit is, for example, the use of a labelled peptide according to the invention as immunochemical reagent in a competition reaction with a HCV antigen to be detected for a binding site on the antibody directed against HCV, which is coated to a solid support.

It is within the scope of this invention to use the new nucleotide sequence or part(s) thereof coding for the amino acid sequence(s) according to the invention, so-called primers, as basis of a test to detect HCV DNA or RNA by a nucleic acid amplification technique for instance the polymerase chain reaction (PCR) or the nucleic acid sequence based amplification

(NASBA), as described in USP 4,683,202 and EP 329,822, respectively.

A test amplification kit for carrying out an amplification and detection method described above is also part of the present invention.

Moreover, a peptide or fragment thereof according to the invention can be used in suitable pharmaceutical dosage forms in the prevention and/or treatment of NANB Hepatitis-disease. The preparation of vaccines thus obtained using such a peptide or fragment thereof as active ingredients, can be accomplished by one skilled in the art.

Description of the Figures:

Figure 1 is a graph illustrating the binding specificy of a monoclonal antibody (HCVHU.OT3 as described in co-pending and co-owned application No. EP 92.204.107.4, the contents of which are incorporated herein by reference) for the NS3 protein.

Figure 2 is a photograph of a recombinant immunoblot assay. The characters A-G represent the following proteins:

A: High Ig control
D: c33c (NS-3)
B: 5-1-1 (NS-4)
E: c22-3 (core)
C: c100-3 (NS-4)
F: superoxide dismutase
G: low Ig control.

Lane 1 represents negative control serum,

Lane 2 represents an anti NS-3 monoclonal antibody (HCVHU.OT3 as described in co-pending and co-owned application No. EP 92.204.107.4).

Lane 3 represents an anti-core monoclonal (HCVHU.OT2), while lane 4 represents polyclonal serum from a HCV-infected patient.

Figure 3 gives a schematic representation of a competition assay performed to determine competition between antibodies in human sera and HCVHU.OT3.

The specific C33 epitope is represented by the triangle, while a cross reactive site on the C33 is represented by the square.

The present invention is further exemplified by the following examples:

Example 1 further exemplifies the specific immune reactivity of monoclonal antibody HCVHU.OT3.

Example 2:

In sub A the construction and screening of a lambda gt-11 library is described, generating the peptides according to the invention.

In sub B the isolation of phage lambda coded beta-galactosidase HCV (C33 derived) hybrid proteins reactive with human sera and a human monoclonal antibody (HCVHU.OT3) is described.

Example 3 illustrates the specificity of HCVHU.OT3..

In Example 4 the specific reactivity of the peptides according to the invention with antibodies directed against HCV is exemplified by showing correlation with PCR results and ELISA results using the peptides according to the invention.

EXAMPLES

Example 1: Testing on anti-NS3 production of B-cell lines.

The specificity of oligoclonal and monoclonal IgG-containing supernatants was further tested with the following reagents:

1) A recombinant purified HCV nucleoprotein expressed in E.coli (Organon Teknika).

2) A recombinant purified NS3 protein expressed in E.coli (Organon Teknika).

3) A recombinant purified NS-5 protein expressed in E.coli.

4) Recombinant immunoblot assay (RIBA II generation, Ortho Diagnostics). This is a nitrocellulose-based assay that includes 4 recombinant HCV antigens:

- c100-3, derived from NS-4 protein,
- 5-1-1, a 42-aminoacid fragment of c100-3,
- c33c, derived from the NS3 protein,
- c22-3, derived from the viral nucleoprotein.

Human superoxide dismutase (SOD) is also present on nitrocellulose strips as a control.

Results:

Supernatant from clone HCVHU.OT3 was analyzed for specificity in the above described way.

Figure 1 illustrates the specific binding capacity of antibodies according to the invention. The binding of the monoclonal antibody HCVHU.OT3 to different HCV derived proteins is compared with the binding of antibodies specific for HCV core and NS4 proteins respectively. As can be seen from Figure 1, the monoclonal antibody HCVHU.OT3 recognized a recombinant NS3

protein preparation and gave a clear positive reaction in Ortho II generation assay, whereas no binding to recombinant HCV core and NS-5 proteins could be documented. Analysis of HCVHU.OT3 supernatant by recombinant immunoblot (RIBA II generation) revealed clear binding to the c33c polypeptide only, further attesting to the specificity of the mAb as can be seen from figure 2, where lane 2 represents an antibody according to the co-pending and co-owned application No. EP 92.204.107.4 (HCVHU.OT3).

Example 2:

A. Construction and screening of lambda gt-11 library

B. Isolation of phage lambda coded beta-galactosidase HCV (C33 derived) hybrid proteins reactive with human sera and a human monoclonal antibody (HCVHU.OT3)

ad A.: The sequence coding for a part of the NS-3 gene of HCV (nucleotides 3573-4890) has been multiplied by PCR using specific primers. Starting material was obtained from a clone constructed by rt-PCR from chimpanzeeserum infected with the prototype HCV-strain. The PCR products were isolated from TBE-polyacrylamide gel (8% PAGE) by electroelution. Portions (20 µl out of 80 µl) of this PCR-material have been digested under controlled conditions (25 °C, 10-60 minutes, in an endvolume of 25 µl containing 1 mM $MnCl_2$, 20 mM Tris-HCl (pH 7.5) and DNAse-1 (Worthington 2635 units per mg, end concentration: 0.6 units). The digestions were stopped in phenol/chloroform-isoamylalcohol and extracted. The DNAse digestions were controlled by nicktranslation. Fragments with the length

from approximately fifty till twohundred basepairs were isolated after 8% PAGE by diffusion (J.Sambrook, E.F.Fritsch, T.Maniatis, Molecular Cloning, second edition, Cold Spring Harbor Laboratory Press, 1989). The polynucleotide, which codes for the peptide according to SEQ ID No. 7 (an identical approach is used for the peptides with respectively SEQ.ID. 6), was tailed with oligo-dG following recommendations of the supplier (GIBCO/BRL). PCR was performed on the tailed product using as a primer poly-C with a terminal EcoR1-site attached. After EcoR1 digestion and phenol extraction the products were cloned in lambda gt-11 arms and transfected into E.coli as detailed by the supplier (Promega). PCR on the libraries using lambda gt11-primers revealed smears with lengths concordant with the lengths of the inserted fragments. The libraries were screened on duplo filters using standard procedures with human monoclonal antibody (1:50) of which the reaction was detected with alkaline phosphatase-conjugated goat anti-human IgG. The positive phages were rescreened to positively identify their contents and thereafter their inserts were transferred to the vector pGEM7Zf(+), (Promega). The inserts in this vector were sequenced using a commercial kit (Pharmacia T7-sequencing kit) according to the recommendations of the supplier.

ad B.: The inserts of the positive phages were also challenged in a Western-blot assay with a choice of sera characterized with commercial tests. For this purpose lytic growth was performed as follows: the inoculum was a single plaque eluted in 1 ml of SM buffer (100mM NaCl, 8mM MgSO4*7H2O, 50 mM Tris-HCl, pH 7.5, 0.01%

gelatin). From this 50 µl was added to 4 ml LB-medium (10 g/l Bacto-tryptone, 5 g/l yeast extract, 5 g/l NaCl), 4 µl ampicillin (50 mg/ml) and 200 µl of a twice concentrated Y1090 overnight culture solved in 10 mM MgSO4.

This mixture was incubated for two hours at 37 °C and mixted by 250 rpm, then the beta-galactosidase fusion protein production was induced with an endconcentration of 10mM IPTG (isopropyl-ß-D-thiogalactopyranoside, Boehringer) and continued for three hours. The culture was centrifuged for five minutes, 12000g in an Eppendorf centrifuge and the pellet solved in 100 µl samplebuffer (62.5 mM Tris-HCl,pH 6.8, 2% SDS, 10% glycerol, 0.01% bromophenolblue) and boiled for five minutes. From this 5 µl was analysed by Western-blotting following standard procedures.(J.Sambrook, E.F.Fritsch, T.Maniatis, Molecular Cloning, second edition, Cold Spring Harbor Laboratory Press, 1989).

The results with sera positive in one or several commercial HCV tests and therefore containing antibodies against C33 are shown in Table 1. As a control sera negative in these tests were included.

The sequences of the clones are given in SEQ.ID. 6 and 7.

Furthermore the sequence as depicted in SEQ.ID. 2 (encoding a peptide comprising the amino acid sequence as depicted in SEQ.ID. 7) was amplified by PCR using primers specific for the ends of this fragment. The PCR fragments were digested under controlled conditions (25 °C, three, five and ten minutes, in an endvolume of 25 microliter containing 1 mM MnCl2, 20 mM Tris-HCl, pH 7.5) and DNAse-1 (Worthington 2635 units per mg, end concentration: 0.6 units). The

digestions were stopped in phenol/chloroform-isoamylalcohol and extracted. The fragments were tailed with oligo-dG following recommendations of the supplier (0.5 µl of DNA with 2 µl of 5* TdT buffer, 2 µl of 1 mM dGTP, 5.2 µl of H2O, 0.3 µl of terminal transferase, 5 units/µl; Gibco/BRL). Fractions containing fragments with a length from approximately hundred till twohundred and fifty basepairs as controlled by electrophoresis were pooled. PCR was performed on the tailed product using as a primer poly-C with a terminal EcoR1-site attached. After EcoR1 digestion and phenol extraction the products were cloned in lambda gt-11 arms and transfected into E.coli as detailed by the supplier (Promega). PCR on the libraries using lambda gt11-primers revealed smears with lengths concordant with the lengths of the inserted fragments. The libraries were screened on duplo filters using standard procedures with the same patientserum (diluted 1:1000) containing anti-HCV (C33) antibodies as determined by several commercial tests as described for the isolation of SEQ.ID. 1 and 2 (encoding a peptide comprising the amino acid sequence as depicted in SEQ.ID. 6 and 7 respectively). The reaction to the immobilized phage-encoded proteins was detected with alkaliphosphatase-conjugated goat anti-human IgG. The positive phages were rescreened to positively identify their contents and thereafter their inserts were transferred to the vector pGEM7Zf(+),(Promega). The inserts in this vector were sequenced using a commercial kit (Pharmacia T7-sequencing kit) according to the recommendations of the supplier.

Spots were made of singly isolated phages on a lawn of host cells. These were immunoscreened

under standard conditions using an array of sera of HCV negative- and positive patients as determined by several commercial tests The results are shown Table 2.

Also immunoscreening by Western-blotting was performed on lysates of the positive phages. Lytic growth was performed as follows: the inoculum was a single plaque eluted in 1 ml of SM buffer (100mM NaCl, 8mM MgSO4*7H2O, 50 mM Tris-HCL, pH 7.5, 0.01% gelatin). From this 50 µl was added to 4 ml LB-medium (10 g/L Bacto-tryptone, 5g/L yeast extract, 5 g/L NaCl), 4 µl ampicillin (50mg/ml) and 200 µl of a twice concentrated Y1090 overnight culture solved in 10 mM MgSO4. This mixture was incubated for two hours at 37°C and mixed by 250 rpm, then the beta-galactosidase fusion protein production was induced with an endconcentration of 10mM IPTG (isopropyl-ß-D-thiopgalactopyranoside; Boehringer) and continued for three hours. The culture was centrifuged for five minutes 12.000g in an Eppendorf centrifuge and the pellet solved in 100 µl samplebuffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 0.01% bromophenolblue) and boiled for five minutes. From this 5 µl was analysed by Western-blotting following standard procedures ((J.Sambrook, E.F.Fritsch, T.Maniatis, Molecular Cloning, second edition, Cold Spring Harbor Laboratory Press, 1989). The sera (dilution 1:100) with which these immunoblots were challenged were positive in one or several commercial HCV tests , while as a control sera (dilution 1:100) negative in these tests were used. The results of this analysis documented in Table 3.

ad. A and ad. B: ß-Galactosidase fusion proteins encoded by the recombinants, obtained as described above, were purified according to standard procedures using anti-ß-galactosidase affinity columns. These purified antigens were coated onto Enzym-Linked Immuno Sorbent Assay (ELISA)-plates and allowed to react with sera from patients with non-A, non-B hepatitis. The use of this ELISA allowed us to discriminate between these patients sera and the normal human serum controles. The procedure is further detailed below.

The peptide according to the sequence of SEQ ID No. 7 (an identical approach is used for the peptides with SEQ.ID. 6, 8-10) is dissolved to 7.5 µg/ml in 100 mM phosphate buffer pH 9.6 and 135 µl of the above peptide solution is placed into each well of a NUNC microtiter plate. Binding of the peptide to the microtiter plate is allowed to proceed overnight at 4^{o} C.

Subsequently the plates are blocked with a solution of 0.05% Tween $20^{(R)}$ in 0.2 M Tris pH 7.4/0.2 M NaCl for 5 min. at room temperature. Plates are then washed once with 0.2 M Tris pH 7.4/0.2 M NaCl, twice with 0.04 M Tris pH 7.4, at 250 µl per well and dried. For the determination of antibodies specific for Non-A, Non-B Hepatitis virus, the serum samples are diluted in sample diluent (phosphate buffered saline (PBS)/20% normal goat serum/1% Triton X100) pipetted into the well (100 µl per well) and incubated for 1 h at 37 ^{o}C. After washing the wells with PBS/0.05% Tween $20^{(R)}$ the bound human antibodies are detected with goat anti-human immunoglobulin labeled with peroxidase (100 µl per well, 1 h at 37 ^{o}C) diluted in sample diluent. The plates are washed 4 times with PBS/0.05% Tween $20^{(R)}$. TMB is

added (100 µl per well) as a substrate for the peroxidase enzyme and the reaction is allowed to proceed for 30 min. at room temperature. The reaction is stopped by adding 100 µl 2M H_2SO_4 to each well. The yellow color is read at 450 nm in an Organon Teknika microelisa reader.

With sera from patients with Non-A Non-B hepatitis positive results can be obtained whereas the results of 20 normal human sera are negative.

As a control, the procedure can be repeated with two unrelated peptides. In both cases no significant differences can be observed in the specific recognition obtained with normal human sera and serum samples from patients with NANBH. From above-mentioned results the conclusion seems justified that said polypeptides according to the invention are extremely immunochemically reactive with HCV-antibodies and can be used alone or in combination in a diagnostic test kit.

With sera from patients with Non-A Non-B hepatitis the specific recognition is positive whereas the results of 20 normal human sera is negative.

Table 1:

CODE DATE	ORTHO 2.0	ORTHO 3.0	LIA -III C1	C2	NS1	NS3	NS4	NS5	LIA tot	RIBA 511	C100	C33	C22	RIBA tot	SEQ.ID.6	SEQ.ID.7
A586 11/15/89	3.84	4.82	3	4	+/-	2	3	4	+	4	4	4	4	+	-	+
A587 09/26/89	3.83	5.02	4	4	2	3	2	4	+	2	4	4	4	+	+	+
A590 03/10/89	3.73	4.75	2	2	+/-	3	4	4	+	4	4	4	4	+	-	-
A597 01/01/89	3.92	3.76	3	2	2	1	2	0	+	2	3	3	4	+	-	-
A598 01/01/89	3.88	3.87	4	4	1	3	4	0	+	4	4	4	4	+	+	+
A599 09/14/89	3.86	4.01	4	4	4	2	2	0	+	1	2	3	3	+	-	+
A604 04/10/89	3.64	3.90	4	1	0	3	4	1	+	4	4	4	4	+	-	-
A653 10/06/89	3.73	4.35	1	2	0	3	4	2	+	0	4	4	4	+	-	-
A717 05/31/90	3.46	4.41	3	1	0	4	+/-	0	+	0	0	4	4	+	+	+
A737 01/11/90	3.41	4.09	4	3	+/-	2	4	0	+	4	1	4	4	+	-	-
A978 10/08/91	1.12	4.22	0	0	0	0	0	0	-	0	0	2	0	IND	-	-
A986 07/10/91		4.50	3	4	+/-	1	+/-	+/-	+	0	0	4	4	+	-	+
H155 04/15/91	3.38	4.29	3	4	0	3	1	+/-	+	2	0	4	4	+	-	-
H180 08/16/91	3.24	4.77	4	4	1	2	0	0	+	0	0	4	4	+	-	-
U027 02/19/92	3.75	4.44	0	0	0	3	4	4	+	4	4	4	0	+	+	+
N207 03/21/88		0.10	0	0	0	0	0	0	-	0	0	0	0	-	-	-
N210 03/28/88		0.07	0	0	0	0	0	0	-	0	0	0	0	-	-	-

Table 1: Reactivity of sera from several at random chosen patients with non-A, non-B hepatitis as tested in 3 commercial assays, compared with their reactivity with the peptides depicted in SEQ.ID. 6 and 7 according to the invention.

Ortho 2.0, Ortho 3.0, LIA-III and RIBA are commercial available HCV test systems.

Abbreviations: Code DATA: Patient sera (sera starting with N are normal human sera)
The figures (scores) defines the reactivity-stages starting from no reaction (0) to the stage of strong reaction (4).

SUBSTITUTE SHEET

Table 2:

CODE	DATE	ORTHO 2.0	ORTHO 3.0	LIA -III C1	C2	NS1	NS3	NS4	NS5	LIA tot	RIBA 511	C100	C33	C22	RIBA tot	SEQ.ID.7	SEQ.ID.8	SEQ.ID.9	SEQ.ID.10
A586	11/15/89	3.84	4.82	3	4	+/-	2	3	4	+	4	4	4	4	+	+	+	-	-
A590	03/10/89	3.73	4.75	2	2	+/-	3	4	4	+	4	4	4	4	+	+	+	+	-
A598	01/01/89	3.88	3.87	4	4	1	3	4	0	+	4	4	4	4	+	+	+	-	-
A604	04/10/89	3.64	3.90	4	1	0	3	4	1	+	4	4	4	4	+	+	+	+	+
A978	10/08/91	1.12	4.22	0	0	0	0	0	0	-	0	0	2	0	IND	-	-	-	-
U027	02/19/92	3.75	4.44	0	0	0	3	4	4	+	4	4	4	0	+	+	+	+	+
N202	03/24/88		0.06	0	0	0	0	0	0	-						-	-	-	-
N203	06/02/88		0.10	0	0	0	0	0	1	IND						-	-	-	-
N205	06/12/88		0.12	0	0	0	0	0	0	-						-	-	-	-

Table 2: Reactivity of sera from several at random chosen patients with non-A, non-B hepatitis as tested in 3 commercial assays, compared with their reactivity with the peptides depicted in SEQ.ID. 7, 8, 9 and 10 according to the invention.

Ortho 2.0, Ortho 3.0, LIA-III and RIBA are commercial available HCV test systems.

Abbreviations:

Code DATA: Patient sera (sera starting with N are normal human sera)
The figures (scores) defines the reactivity-stages starting from no reaction (0) to the stage of strong reaction (4).

SUBSTITUTE SHEET

Table 3:

CODE DATE	ORTHO 2.0	ORTHO 3.0	LIA -III C1	C2	NS1	NS3	NS4	NS5	LIA tot	RIBA 511	C100	C33	C22	RIBA tot	SEQ.ID.7	SEQ.ID.8	SEQ.ID.9
A586 11/15/89	3.84	4.82	3	4	+/-	2	3	4	+	4	4	4	4	+	+	+	-
A590 03/10/89	3.73	4.75	2	2	+/-	3	4	4	+	4	4	4	4	+	+	+	+
A598 01/01/89	3.88	3.87	4	4	1	3	4	0	+	4	4	4	4	+	+	+	-
A604 04/10/89	3.64	3.90	4	1	0	3	4	1	+	4	4	4	4	+	+	+	+
A978 10/08/91	1.12	4.22	0	0	0	0	0	0	-	0	0	2	0	IND	-	-	-
U027 02/19/92	3.75	4.44	0	0	0	3	4	4	+	4	4	4	0	+	+	+	+
N202 03/24/88		0.06	0	0	0	0	0	0	-						-	-	-

Table 3: Reactivity of sera from several at random chosen patients with non-A, non-B hepatitis as tested in 3 commercial assays, compared with their reactivity with the peptides depicted in SEQ.ID. 7, 8 and 9 according to the invention.

Ortho 2.0, Ortho 3.0, LIA-III and RIBA are commercial available HCV test systems.

Abbreviations:

Code DATA: Patient sera (sera starting with N are normal human sera)
The figures (scores) defines the reactivity-stages starting from no reaction (0) to the stage of strong reaction (4).

Example 3: Hu OT3 competition assay.

Recombinant C33 antigen (Chiron, EP 318.216) was purified and coated on ELISA plates in carbonate buffer pH 9.6 at a concentration of 1µg/ml. HuOT3 (which is the antibody according to the our co-owned and copending application EP 92.204.107.4) was labelled with HRP using standard procedures.

In brief: culture supernatant was applied to a protein-A-Sepharose column at AT. The column was washed (3M NaCl 1.5 M Glycine pH 8.9) and subsequently eluted using a 0.1 M citric acid buffer with 4 M NaOH at pH 4.0. Fractions were collected in saturated Tris to a final pH of 7.0. Fractions were concentrated on an Amicon microconcentrator and desalted on a PD-10 column. IgG concentrations were determined using a micro-BCA-assay. Monoclonal antibodies were conjugated to HRP using the Nakane conjugation method in a molar ratio of HRP:IgG = 4:1.

To determine whether sera from HCV patients contain antibodies that compete with HuOT3, ELISA plates coated with C33 recombinant antigen were incubated with a mixture of that human serum, 10 times diluted in PBS/Tween 20 and an appropriate dilution of HRP labelled HuOT3 that would result in an A492 of approximately 1.0 (C_t).

The schematic representation of the competition assay is shown in figure 3. The specific C33 epitope is represented by a triangle, while a cross reactive site on the C33 is represented by the square. C33-only sera that do recognize C33 but are PCR negative may contain antibodies to the "square" cross reacting epitope as presented in figure 3.

A microtiter well of the above described C33 ELISA was preincubated with the particula-

unlabelled competing serum under investigation. After 30 min., the HRP labelled HuOT3 was added and both the labelled and the unlabelled antibodies were allowed to compete for binding for another 30 minutes. To determine 100% competition, the human monoclonal was used in a 100-fold molar excess.

The following parameters were determined:

A: A492 obtained when a particular HRP labelled monoclonal antibody was tested at C_t without competing antibody present: 0% competition.

B: A492 obtained when the microtiter well was preincubated with the same monoclonal antibody (unlabelled) in a 100-fold excess: 100% competition.

C: A492 obtained when the microtiter well was preincubated with the competing antibody (unlabelled).

The rate of competition of a particular competing serum was calculated as follows:

% competition = [1-(C-B)/(A-B)] x 100%

This assay was validated with 10 normal control sera and 10 sera from HCV patients that were found PCR positive and RIBA positive.

All PCR positive sera competed with the HuOT3 antibody, while the control sera did not, form which it can be concluded that the HuOT3 antibody recognizes an important, immunodominant epitope on the NS-3 antigen.

With the competition assay, using the procedure as described, 79 C33-only sera were tested. results obtained were compared with the results obtained with these sera in a PCR test. From these 79 sera 11 sera were PCR positive,

while 68 sera were PCR negative. All (except one) PCR negative sera did not compete with the HuOT3 antibody in the competition assay, while all PCR positive sera did compete with the HuOT3 antibody in the competition assay. Results of this comparison are presented in Table 4.

Table 4: Correlation between results obtained with competition assay and PCR test.

	COMPETITION YES	NO
PCR POS	11	0
PCR NEG	1	67

Example 4: ELISA with cloned epitope.

The ability to discriminate between C33 antibodies from PCR-positive and PCR-negative sera with an ELISA based on the use of the peptide, having the amino acid sequence as depicted in SEQ ID No.: 7 is further illustrated in this example.

A clone comprising the nucleic acid sequence encoding the peptide with the sequence as depicted in SEQ ID No.:7 was expressed as a ß-galactosidase fusion protein by lysogenic lambda-gt11 phages. The fusion protein was purified using an anti-ß-gal affinity column (Promega) according to the manufactures instructions. The purified fusion protein was diluted to 1 µg/ml in carbonate buffer and coated on microtiter ELISA plates. Sera were diluted 1:34 and tested using standard ELISA procedures. Binding of specific

antibody was determined with a goat-anti-human peroxidase conjugate. Cut-off value was statistically determined as average value obtained with 20 normal sera plus 5 times the standard deviation.

30 C33-only sera were tested in the above described way and results were compared to results obtained using a C33 RIBA (Recombinant immunoblot assay). Results are listed in Table 5; As can be seen form this table the RIBA antigen did not discriminate between PCR positive and PCR negative sera and detected specific as well as non-specific antibodies. The OT3 antigen identified 9 out of 9 PCR positive sera and reacted with only 1 out of 21 PCR negative sera.

Table 5: Specificity of HuOT3 epitope.

	C33 RIBA		OT3 epitope	
	pos	neg	pos	neg
PCR pos	9	0	9	0
PCR neg	21	0	1	20

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:
(A) NAME: AKZO N.V.
(B) STREET: Velperweg 76
(C) CITY: Arnhem
(D) COUNTRY: The Netherlands
(E) POSTAL CODE (ZIP): 6824 BM

(ii) TITLE OF INVENTION:
Peptides from the C33-region of HCV, antibodies thereto and methods for the detection of HCV

(iii) NUMBER OF SEQUENCES: 10

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: EP 92203802
(B) FILING DATE: 07-DEC-1992

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 241 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Escherichia Coli
(B) STRAIN: JM101

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

AGGAGGTTGC TCTGTCCACC ACCGGAGAGA TCCCTTTTTA 40
CGGCAAGGCT ATCCCCCTCG AAGTAATCAA GGGGGGGAGA 80
CATCTCATCT TCTGTCATTC AAAGAAGAAG TGCGACGAAC 120
TCGCCGCAAA GCTGGTCGCA TTGGGCATCA ATGCCGTGGC 160
CTACTACCGC GGTCTTGACG TGTCCGTCAT CCCGACCAGC 200
GGCGATGTTG TCGTCGTGGC AACCGATGCC CTCATGACCG 240
G 241

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 274 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Escherichia coli
(B) STRAIN: JM101

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

AGGAGGTTGC TCTGTCCACC ACCGGAGAGA TCCCTTTTTA 40
CGGCAAGGCT ATCCCCCTCG AAGTAATCAA GGGGGGGAGA 80
CATCTCATCT TCTGTCATTC AAAGAAGAAG TGCGACGAAC 120
TCGCCGCAAA GCTGGTCGCA TTGGGCATCA ATGCCGTGGC 160
CTACTACCGC GGTCTTGACG TGTCCGTCAT CCCGACCAGC 200
GGCGATGTTG TCGTCGTGGC AACCGATGCC CTCATGACCG 240
GCTATACCGG CGACTTCGAC TCGGTGATAG ACTG 274

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 201 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Escherichia coli
(B) STRAIN: JM101

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

GGGAGACATC TCATCTTCTG TCATTCAAAG AAGAAGTGCG 40
ACGAACTCGC CGCAAAGCTG GTCGCATTGG GCATCAATGC 80
TGTGGCCTAC TACCGCGGTC TTGACGTGTC CGTCATCCCG 120

ACCAGCGGCG ATGTTGTCGT CGTGGCAACC GATGCCCTCA 160
TGACCGGCTA TACCGGCGAC TTCGACTCGG TGATAGACTG 200
C 201

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 195 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Escherichia coli
(B) STRAIN: JM101

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

CATCTCATCT TCTGTCATTC AAAGAAGAAG TGCGACGAAC 40
TCGCCGCAAA GCTGGTCGCA TTGGGCATCA ATGCTGTGGC 80
CTACTACCGC GGTCTTGACG TGTCCGTCAT CCCGACCAGC 120
GGCGATGTTG TCGTCGTGGC AACCGATGCC CTCATGACCG 160
GCTATACCGG CGACTTCGAC TCGGTGATAG ACTGC 195

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 153 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Escherichia coli
(B) STRAIN: JM101

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

GCCGCAAAGC TGGTCGCATT GGGCATCAAT GCTGTGGCCT 40
ACTACCGCGG TCTTGACGTG TCCGTCATCC CGACCAGCGG 80
CGATGTTGTC GTCGTGGCAA CCGATGCCCT CATGACCGGC 120
TATACCGGCG ACTTCGACTC GGTGATAGAC TGC 153

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 79 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

Glu Val Ala Leu Ser Thr Thr Gly Glu Ile
1 5 10

Pro Phe Tyr Gly Lys Ala Ile Pro Leu Glu
15 20
Val Ile Lys Gly Gly Arg His Leu Ile Phe
25 30
Cys His Ser Lys Lys Lys Cys Asp Glu Leu
35 40
Ala Ala Lys Leu Val Ala Leu Gly Ile Asn
45 50
Ala Val Ala Tyr Tyr Arg Gly Leu Asp Val
55 60
Ser Val Ile Pro Thr Ser Gly Asp Val Val
65 70
Val Val Ala Thr Asp Ala Leu Met Thr
75 79

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 90 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

Glu Val Ala Leu Ser Thr Thr Gly Glu Ile
1 5 10
Pro Phe Tyr Gly Lys Ala Ile Pro Leu Glu
15 20
Val Ile Lys Gly Gly Arg His Leu Ile Phe

25 30
Cys His Ser Lys Lys Lys Cys Asp Glu Leu
35 40
Ala Ala Lys Leu Val Ala Leu Gly Ile Asn
45 50
Ala Val Ala Tyr Tyr Arg Gly Leu Asp Val
55 60
Ser Val Ile Pro Thr Ser Gly Asp Val Val
65 70
Val Val Ala Thr Asp Ala Leu Met Thr Gly
75 80
Tyr Thr Gly Asp Phe Asp Ser Val Ile Asp
85 90

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 67 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

Gly Arg His Leu Ile Phe Cys His Ser Lys
1 5 10
Lys Lys Cys Asp Glu Leu Ala Ala Lys Leu
15 20
Val Ala Leu Gly Ile Asn Ala Val Ala Tyr
25 30

Tyr Arg Gly Leu Asp Va' Ser Val Ile Pro
35 40
Thr Ser Gly Asp Val Val Val Val Ala Thr
45 50
Asp Ala Leu Met Thr Gly Tyr Thr Gly Asp
55 60
Phe Asp Ser Val Ile Asp Cys
65

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 65 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

His Leu Ile Phe Cys His Ser Lys Lys Lys
1 5 10
Cys Asp Glu Leu Ala Ala Lys Leu Val Ala
15 20
Leu Gly Ile Asn Ala Val Ala Tyr Tyr Arg
25 30
Gly Leu Asp Val Ser Val Ile Pro Thr Ser
35 40
Gly Asp Val Val Val Val Ala Thr Asp Ala
45 50
Leu Met Thr Gly Tyr Thr Gly Asp Phe Asp

55 60

Ser Val Ile Asp Cys
65

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 51 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

Ala Ala Lys Leu Val Ala Leu Gly Ile Asn
1 5 10
Ala Val Ala Tyr Tyr Arg Gly Leu Asp Val
15 20
Ser Val Ile Pro Thr Ser Gly Asp Val Val
25 30
Val Val Ala Thr Asp Ala Leu Met Thr Gly
35 40
Tyr Thr Gly Asp Phe Asp Ser Val Ile Asp
45 50
Cys

Claims

1. A peptide with amino acid sequence depicted in SEQ.ID. No.: 7, or fragments thereof or analogues thereof which are immunochemically reactive with HCV-antibodies.

2. A peptide with amino acid sequence selected from the group of sequences depicted in SEQ.ID. 6, 8, 9, 10, and combinations thereof or fragments of said group of sequences or analogues of said group of sequences which are immunochemically reactive with HCV-antibodies.

3. Immunochemical reagent comprising a peptide according to any of claims 1-2.

4. A nucleic acid sequence encoding a peptide according to any of claims 1-2.

5. A nucleic acid sequence according to claim 4, containing at least part of the DNA sequence shown in SEQ.ID. No. 2.

6. A nucleic acid sequence according to claims 4, containing at least part of the DNA sequence shown in SEQ.ID. No. 1, 3, 4, 5.

7. A recombinant vector molecule comprising a nucleic acid sequence according to any of claims 4-6.

8. A host cell transformed with a vector according to claim 7.

9. A monoclonal antibody directed against a peptide according to any of claims 1-2.

10. Immortalized cell line capable of excreting antibodies according to claim 9.

11. Immunodiagnostic reagent comprising a monoclonal antibody according to claim 9.

12. Method for the detection of antibodies directed against HCV in a test fluid, wherein a peptide according to any of claims 1-2 is brought into contact with the test fluid and the presence of immune complexes formed between the peptide and antibodies in the test fluid is detected.

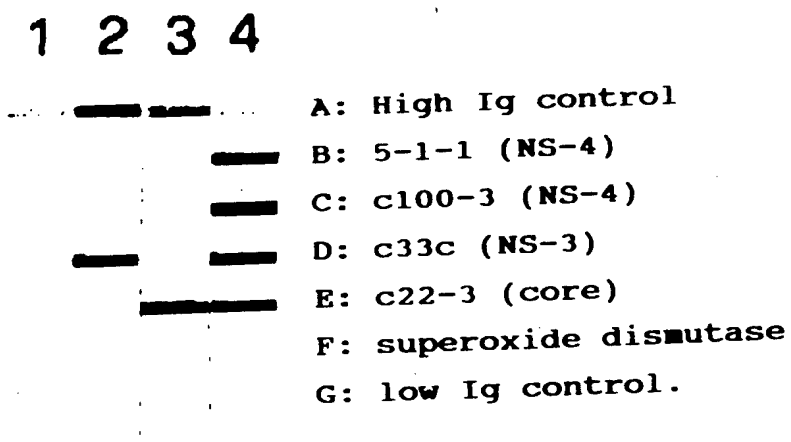
13. Method for the detection of hepatitis C virus in a sample comprising contacting the sample with a monoclonal antibody according to claim 9, and detecting immune complexes formed between the monoclonal antibody and a hepatitis C antigen.

14. Method for the detection of antibodies directed against a HCV specific epitope of the NS-3 protein of the Hepatitis C virus in a test-fluid, wherein a peptide according to claim 1 or 2 is brought into contact with the test fluid and the presence of immune complexes formed between the peptide and the antibodies in the test fluid is detected.

15. Method for the detection of antibodies directed against a HCV specific epitope of the C33 protein of the Hepatitis C virus in a test-fluid, wherein a peptide according to claim 1 or 2, coated on a suitable support, is brought into contact with the test fluid and with an antibody

according to claim 9, provided with a label and any label bound to the solid phase is detected.

Figure 2/3



Lane 1: negative control serum
Lane 2: anti NS-3 monoclonal antibody (HCVHU.OT3)
Lane 3: anti-core monoclonal antibody (HCVHU.OT2)
Lane 4: polyclonal serum from a HCV-infected patient.

Figure 1/3

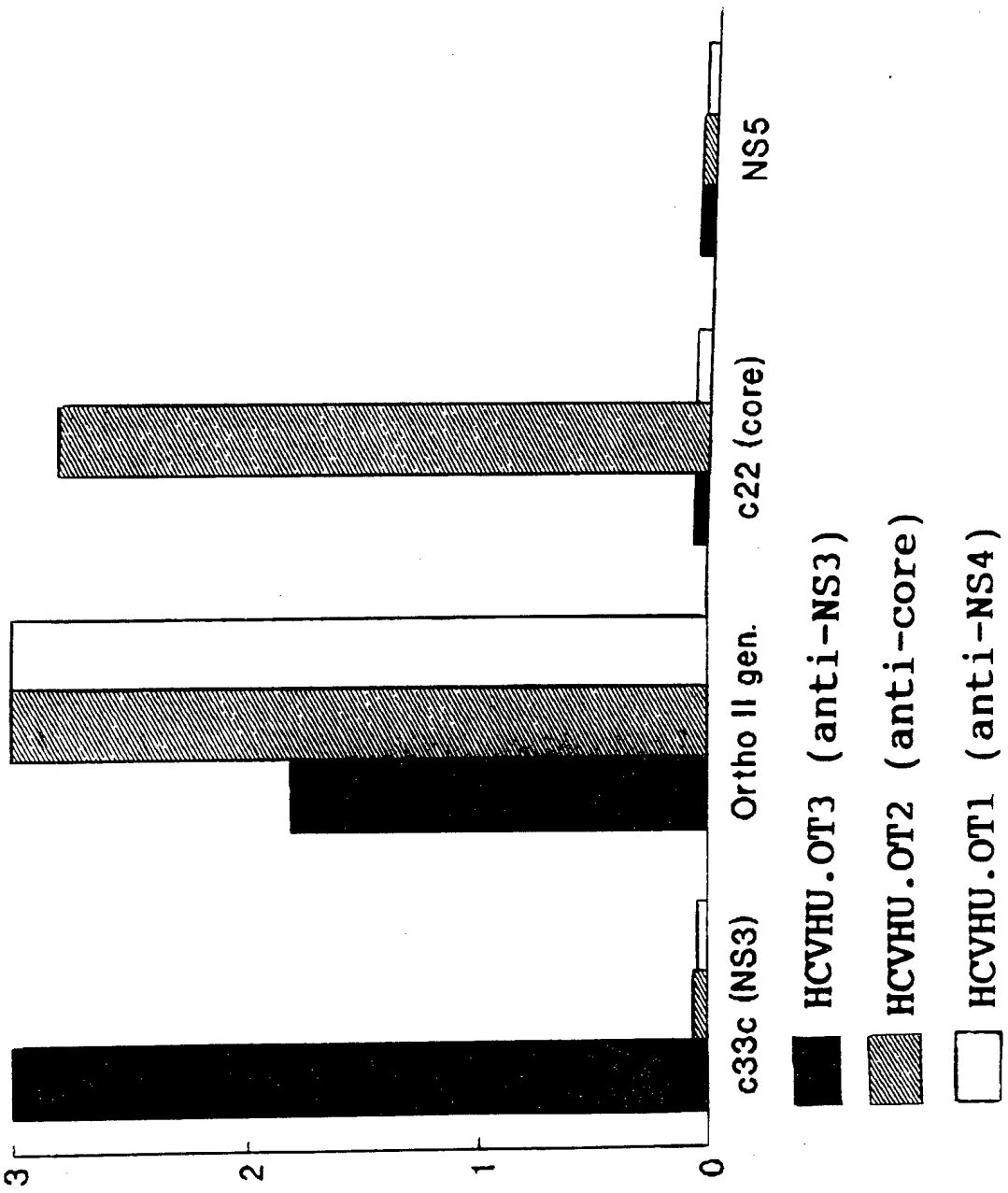
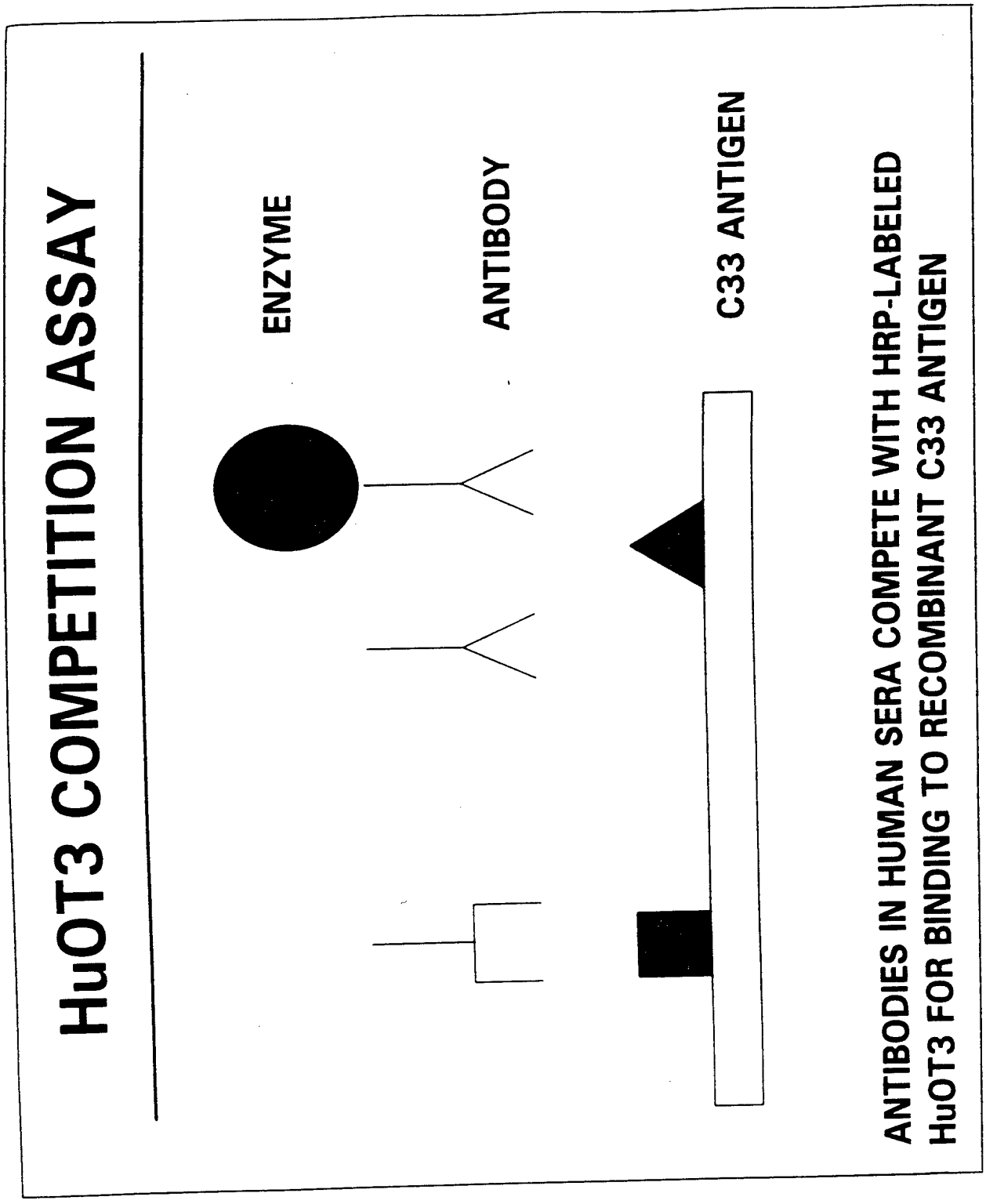

3
2
1
0
c33c (NS3)
Ortho II gen.
c22 (core)
NS5
HCVHU.OT3 (anti-NS3)
HCVHU.OT2 (anti-core)
HCVHU.OT1 (anti-NS4)

Figure 3/3


HuOT3 COMPETITION ASSAY
ENZYME
ANTIBODY
C33 ANTIGEN
ANTIBODIES IN HUMAN SERA COMPETE WITH HRP-LABELED HuOT3 FOR BINDING TO RECOMBINANT C33 ANTIGEN

INTERNATIONAL SEARCH REPORT

International application No.
PCT/EP 93/03478

A. CLASSIFICATION OF SUBJECT MATTER
IPC 5 C07K15/00 G01N33/576 C12N15/40 C12N15/51 C12N5/10
C12P21/08 C12Q1/70 A61K39/29

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 5 C07K G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP,A,0 318 216 (CHIRON CORP) 31 May 1989 see abstract; claims 1-40; figures 14.1,32.3,47	1-15
X	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA., vol.88, 1991, WASHINGTON US pages 2451 - 2455 CHOO Q.L. ET AL. see the whole document	1-8
A		14
X	WO,A,91 15596 (PROTOS INC.) 17 October 1991 see abstract; figure 8; example 3	2,6

☐ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
8 April 1994	20. 05. 94

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Gurdjian, D

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.
PCT/EP 93/03478

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
EP-A-0318216	31-05-89	AU-A-	2796789	14-06-89
		CN-A-	1073719	30-06-93
		DE-D-	3886363	27-01-94
		GB-A-	2212511	26-07-89
		JP-T-	2500880	29-03-90
		WO-A-	8904669	01-06-89
WO-A-9115596	17-10-91	AU-A-	7679491	30-10-91